DESCRIPTION

Method for Detecting Gene Mutation and kit for Detecting Gene Mutation

Technical Field

The present invention relates to a method for detecting a gene mutation and a kit for detecting a gene mutation using the method that are especially useful in the field of bio-informatics (life information science), and more particularly to a method for detecting a gene mutation and a kit for detecting a gene mutation by which single nucleotide substitution in a gene arrangement is simply and rapidly detected.

This application claims a priority based on Japanese Patent Application No. 2004-080703 filed on March 19, 2004 in Japan that is applied to this application with reference thereto.

Background Art

One of the targets of a study after a human genome arrangement is decoded nowadays resides in an identification of a gene, an analysis of a function and the variety of genes for determining a individual difference influenced by the expression or the function of the gene. Here, the solid difference of the gene caused from the difference of single nucleotide in a nucleobase sequence is referred to as a single nucleotide mutation. The mutation having the highest frequency in the single nucleotide mutation is called a Single Nucleotide Polymorphism (SNP). The SNPs dotted in the gene are obviously strongly

related to many kinds of diseases.

Currently, as a method for detecting the gene mutation including the SNPs, an electrophoresis method is exemplified in which a DNA fragment cut by a restriction enzyme is separated by gel, and then, the DNA fragment is colored and detected by a dye. Though this method is generally used, a problem arises that this method requires a long time for separation or coloring so that it is low in its rapidity.

Further, an integrated substrate for a bio-assembly referred to as what is called a DNA chip in which prescribed DNAs are finely arranged by a micro-array technique begins to be used for detecting a gene mutation. In this DNA chip, since many various kinds of DNA oligonucleotide chains or cDNA or the like are accumulated on a glass substrate or a silicon substrate, many genes can be inspected at one time. The DNA chip is anticipated to be applied to a clinical laboratory field. However, since the DNA chip is based on a method using as a principle the stability of a double-stranded DNA derived from the formation of a mismatch of a nucleobase, it is difficult to control the temperature thereof depending on a base sequence. Further, there is a problem that a pre-process is necessary for modifying an object to be inspected itself by a radioactive material or a fluorescent dye.

Further, in recent years, a real time PCR method in which the object to be inspected is amplified and detected at the same time has been progressively spread

as a technique of a rapid quantitative measurement of one stage by a nucleic acid amplification method. However, since a temperature control is complicated in an amplifying reaction, the design of a primer applied to each gene arrangement including the introduction of a probe is complicated, and further, obtained results are frequently different depending on amplifiers or conditions, a problem still remains in view of reproducibility. Further, since a detection is carried out by using the change of a signal with an elapse of time, an operability is undesirably slightly insufficient.

As described above, the usual technique for detecting the gene mutation requires a precise temperature control or a complicated pre-process of the object to be inspected, or has a problem that a long time is required until a measurement. Therefore, in the usual technique, the gene cannot be simply and rapidly inspected.

Thus, the inventors of this application propose a technique, in the document "K. Yoshimoto, S. Nishizawa, M. Minagawa and N. Teramae, "Use of Abasic Site-Containing DNA Strands for Nucleobase Recognition in Water", J. Am. Chem. Soc., 2003, 125, pp. 8982-8983" (refer this document to as a document 1, hereinafter), in which a double-stranded nucleic acid is formed by a single-stranded target DNA having single nucleotide substitution part and a single-stranded detecting DNA complementary to this target DNA and having an abasic site (AP site) except a corresponding base corresponding to the single nucleotide substitution part, a receptor molecule having hydrogen bonding

characteristics and a fluorescence is added to the double-stranded nucleic acid to form a hydrogen bond to the single nucleotide substitution part, and the change of the fluorescent strength of the receptor molecule is measured to effectively detect the single nucleotide substitution.

Since the technique disclosed in the document 1 does not require, in principle, a complicated operation such as labeling of the target DNA as the object to be inspected or a heat control, the number of processes is extremely small. Further, since the technique does not depend, in principle, on the thermal stability of the double-stranded DNA itself, a very short time is merely necessary until the detection and reproducibility is also excellent. Further, since a visual recognition using a UV lamp can be realized, the detection can be achieved under a state having no special equipment.

However, in the technique disclosed in the document 1, though a chemical modification that the detecting DNA is marked by a fluorescent material is not required, a special part such as the abasic site needs to be introduced, which undesirably corresponds to the chemical modification in a strict sense. Further, since the abasic site is introduced, a problem arises that a cost necessary when the detecting DNA is synthesized is high.

Disclosure of the Invention

The present invention is proposed by considering the above-described usual circumstances, and it is an object of the present invention to provide a

method for detecting a gene mutation and a kit for detecting a gene mutation in which a gene mutation is simply and rapidly detected without performing a chemical modification to a target DNA and a detecting DNA.

In order to achieve the above-described object, a method for detecting a gene mutation according to the present invention comprises: a step of forming a double-stranded nucleic acid by a single-stranded target nucleic acid having a target base composed of one or more continuous bases and two kinds of single-stranded detecting nucleic acids complementary to two kinds of partial sequences that sandwich the target base between them; a step of inserting a receptor having hydrogen bonding characteristics and fluorescence emitting characteristics into the double-stranded nucleic acid to form a hydrogen bond with the target base: and a step of measuring the fluorescent strength of the double-stranded nucleic acid into which the receptor is inserted.

Further, to achieve the above-described object, a kit for detecting a gene mutation according to the present invention comprises: two kinds of single-stranded detecting nucleic acids complementary to two kinds of partial sequences that sandwich a target base between them in a single-stranded target nucleic acid having the target base composed of one or more continuous bases; and a receptor having hydrogen bonding characteristics and fluorescence emitting characteristics and inserted into a double-stranded nucleic acid formed by the target nucleic acid and the two kinds of detecting nucleic acids to form a hydrogen

bond with the target base.

In the above-described method for detecting a gene mutation and the kit for detecting a gene mutation, the double-stranded nucleic acid is formed by the target nucleic acid and the two kinds of detecting nucleic acids to intentionally form a gap part in the double-stranded nucleic acid. The receptor having the hydrogen bonding characteristics and the fluorescence emitting characteristics is added to the double-stranded nucleic acid to insert the receptor into the gap part and form the hydrogen bond with the target base. Then, the fluorescent strength of the double-stranded nucleic acid into which the receptor is inserted is measured to detect a gene mutation generated in the target base.

As the above described receptor, usable are, for instance, a naphthylidine derivative, a quinoline derivative, a pteridine derivative, a coumarin derivative, an indazol derivative, an alloxazine derivative or amyloride.

Here, the receptor may be fixed to a substrate.

That is, to achieve the above-described object, a method for detecting a gene mutation according to the present invention comprises: a step of dropping on a substrate to which a receptor having hydrogen bonding characteristics is fixed a single-stranded target nucleic acid having a target base composed of one or more continuous bases and two kinds of single-stranded detecting nucleic acids complementary to two kinds of partial sequences that sandwich the target base between them to form a double-stranded nucleic acid by the target nucleic acid and

the two kinds of detecting nucleic acids and form a hydrogen bond by the target base and the receptor; and a step of identifying the target base on the basis of the bond of the target base and the receptor.

Further, in order to achieve the above-described object, a kit for detecting a gene mutation according to the present invention comprises: two kinds of single-stranded detecting nucleic acids complementary to two kinds of partial sequences that sandwich a target base between them in a single-stranded target nucleic acid having the target base composed of one or more continuous bases; a receptor having hydrogen bonding characteristics and inserted into a double-stranded nucleic acid formed by the target nucleic acid and the two kinds of detecting nucleic acids to form a hydrogen bond with the target base; and a substrate to which the receptor is fixed.

In the above-described method for detecting a gene mutation and the kit for detecting a gene mutation, the double-stranded nucleic acid is formed by the target nucleic acid and the two kinds of detecting nucleic acids to intentionally form a gap part in the double-stranded nucleic acid. The double-stranded nucleic acid is dropped on the substrate to which the receptor having the hydrogen bonding characteristics is fixed to insert the receptor into the gap part and form the hydrogen bond with the target base. Then, a gene mutation generated in the target base is detected on the basis of the bond of the target base and the receptor. In this case, when the receptor shows fluorescence emitting characteristics, the

target base can be identified on the basis of the change of fluorescent strength of the double-stranded nucleic acid into which the receptor is inserted. Further, the target base can be identified on the basis of the change of a signal strength of a surface plasmon resonance due to the bond of the target base and the receptor or the change of resonance frequency of a crystal oscillator.

Further, one of the two kinds of detecting nucleic acids may be fixed to the substrate.

That is, to attain the above-described object, a method for detecting a gene mutation according to the present invention comprises: a step of dropping on a substrate to which one detecting nucleic acid of two kinds of single-stranded detecting nucleic acids complementary to two kinds of partial sequences that sandwich a target base between them in a single-stranded target nucleic acid having the target base composed of one or more continuous bases is fixed, the target nucleic acid, the other detecting nucleic acid and a receptor showing hydrogen bonding characteristics to form a double-stranded nucleic acid by the target nucleic acid and the two kinds of detecting nucleic acids and form a hydrogen bond by the target base and the receptor; and a step of identifying the target base on the basis of the bond of the target base and the receptor.

Further, to achieve the above-described object, a kit for detecting a gene mutation according to the present invention comprises: two kinds of single-stranded detecting nucleic acids complementary to two kinds of partial

sequences that sandwich a target base between them in a single-stranded target nucleic acid having the target base composed of one or more continuous bases; a receptor having hydrogen bonding characteristics and inserted into a double-stranded nucleic acid formed by the target nucleic acid and the two kinds of detecting nucleic acids to form a hydrogen bond with the target base; and a substrate to which one of the two kinds of detecting nucleic acids is fixed.

In the above-described method for detecting a gene mutation and the kit for detecting a gene mutation, on a substrate to which one detecting nucleic acid of the two kinds of detecting nucleic acids is fixed, the target nucleic acid, the other detecting nucleic acid and the receptor showing the hydrogen bonding characteristics are dropped to form the double-stranded nucleic acid by the target nucleic acid and the two kinds of detecting nucleic acids and intentionally form a gap part in the double-stranded nucleic acid. The receptor is inserted into the gap part to form the hydrogen bond with the target base. Then, a gene mutation generated in the target base is detected on the basis of the bond of the target base and the receptor. In this case, when the receptor shows fluorescence emitting characteristics, the target base can be identified on the basis of the change of fluorescent strength of the double-stranded nucleic acid into which the receptor is inserted.

Other objects of the present invention and specific advantages obtained by the present invention will be more apparent from the following description of

embodiments.

Brief Description of the Drawings

Fig. 1 is a diagram for explaining a principle for detecting a gene mutation in this embodiment.

Fig. 2 is a diagram for explaining a principle for detection a gene mutation when a receptor molecule is fixed to a substrate.

Fig. 3 is a diagram for explaining a principle for detecting a gene mutation when one of detecting nucleic acids is fixed to the substrate.

Fig. 4 is a diagram showing fluorescence spectrum after an AMND is added when target bases are guanine and cytosine.

Fig. 5 is a diagram showing fluorescence after the AMND is added when the target bases are guanine and cytosine.

Fig. 6 is a diagram showing a fluorescence quenching effect after the AMND is added when the target bases are guanine, cytosine, adenine and thymine.

Fig. 7 is a diagram showing a fluorescence quenching effect after DiMe-pteridine is added when the target bases are guanine, cytosine, adenine and thymine.

Fig. 8 is a diagram showing a fluorescence quenching effect after amyloride is added when the target bases are guanine, cytosine, adenine and thymine.

Fig. 9 is a diagram showing a fluorescence quenching effect after the

AMND is added when the target bases of a PCR amplified target nucleic acid are guanine, cytosine, adenine and thymine.

Fig. 10 is a diagram showing the SPR signal strength of a sensor chip to which an AMND-DPA is fixed when the target bases are guanine, cytosine, adenine and thymine.

Fig. 11 is a diagram showing the SPR signal strength of a sensor chip to which an AcMND-C5A is fixed when the target bases are guanine, cytosine, adenine and thymine.

Best Mode for Carrying Out the Invention

Ordinarily, the recognition of a nucleobase using a hydrogen bond has a feature that a high base selectivity can be relatively easily obtained by changing the forms or the number of the hydrogen bonds of a receptor molecule. At this time, since a recognizing function based on the formation of the hydrogen bond cannot be anticipated in a completely aqueous solution, most of usual studies are limited under an environment of a nonpolar solvent as in chloroform, which results in, however, the denaturation and precipitation of a nucleic acid in the solvent.

Thus, in this embodiment, as conceptually shown in Fig. 1, solution including a single-stranded target nucleic acid 10 having a target base 11 related to an SNP is mixed with solution including two kinds of single-stranded detecting nucleic acids 20a and 20b complementary to partial sequences that sandwich the target base 11 between them to hybridize the target nucleic acid 10 with the

detecting nucleic acids 20a and 20b. Thus, a gap part 21 is intentionally formed at a position opposed to the target base 11. Then, a receptor molecule 30 showing hydrogen bonding characteristics is inserted into the gap part 21 as a hydrophobic space to form a hydrogen bond with the target base 11.

As described above, the receptor molecule 30 showing the hydrogen bonding characteristics is inserted into the gap part 21 as the hydrophobic space to form the hydrogen bond with the target base 11. Thus, even in the completely aqueous solution, the nucleobase is effectively recognized, so that the mutation of the target base 11 can be detected.

Further, when the solution including the two kinds of single-stranded detecting nucleic acids 20a and 20b complementary to the partial sequences that sandwich a plurality of bases of the target nucleic acid 10 between them is employed, the mutation of the plurality of bases can be detected. In this case, when, for instance, the two bases correspond to the gap part 21, two receptor molecules 30 are inserted into the gap part 21.

Here, as the target nucleic acid 10 that can be analyzed in this embodiment, for instance, DNA, cDNA or the like originated from Mammalia including human beings or plants may be exemplified, however, the target nucleic acid is not especially limited to specific nucleic acids and is diluted, concentrated and amplified if necessary.

As the receptor molecule 30 showing the hydrogen bonding

characteristics, a reagent having a hydrogen bonding part and showing fluorescence emitting characteristics is desirable. Specifically, a reagent having a heterocyclic aromatic group is preferable that has at least one stage, or preferably, a plurality of stages of hydrogen bonding parts and can stack on the nucleobase adjacent to the gap part 21. Particularly, a water soluble reagent is preferable. However, in the case of a non-water soluble reagent, this reagent may be met by using a small amount of an organic solvent. As such receptor molecule 30, for instance, may be exemplified a naphthylidine derivative, a quinoline derivative, a pteridine derivative, a coumarin derivative, an indazol derivative, an alloxazine derivative or amyloride.

In the above-described embodiment, a liberated target nucleic acid 10 is allowed to react with liberated detecting nucleic acids 20a and 20b in the solution, however, the present invention is not limited thereto.

For instance, as schematically shown in Fig. 2, the receptor molecule 30 may be fixed to a substrate 40 through a linker molecule 41 and the solution including the target nucleic acid 10 and the detecting nucleic acids 20a and 20b may be dropped on the substrate 40. Further, as schematically shown in Fig. 3, the detecting nucleic acid 20a may be fixed to the substrate 40 through the linker molecule 41 and the solution including the target nucleic acid 10, the detecting nucleic acid 20b and the receptor molecule 30 may be dropped on the substrate 40.

In such a way, a sensor chip (a micro-array) having many receptor

molecules 30 or the detecting nucleic acids 20a accumulated on the substrate 40 is manufactured and used as a kit for detecting a gene mutation. Thus, the detection with a high throughput that overcomes usual shortcomings can be realized.

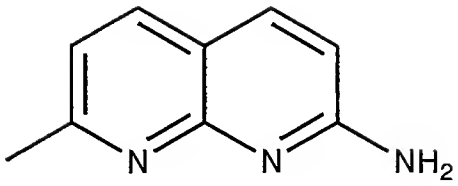
In the case of a structure shown in Fig. 2, a gene mutation can be detected by using not the change of a fluorescent strength, but the change of a signal strength of a surface plasmon resonance (SPR) (for instance, see a document "Kazuhiko Nakatani, Shinsuke Sando, and Isao Saito, Nat. Biotechnol., 2001, 19, pp. 51-55", a document "Akio Kobori, Souta Horie, Hitoshi Suda, Isao Saito, and Kazuhiko Nakatani, J. Am. Chem. Soc., 2004, 126, pp. 557-562".). Further, in the case of the structure shown in Fig. 2, the gene mutation may be detected by using the change of resonance frequency of a crystal oscillator.

Now, specific examples of the present invention will be described below in detail by referring to the drawings. However, the present invention is not limited to the following examples and various changes may be made within a scope without departing the gist of the present invention.

(First Example)

In a first example, as a receptor molecule, 2-amino-7-methyl-1,8-naphthylidine (AMND) as a naphthylidine derivative as shown in a below-described chemical formula was employed. The AMND was synthesized from 2,6-diaminopyridine with reference to a document "E. V. Brown, J. Org. Chem., Vo. 30, pl 607, 1965".

Chemical formula 1



This AMND shows fluorescence emitting characteristics and interacts with a target base when the AMND is inserted into a gap part between two kinds of detecting DNAs as described below. Since the fluorescent strength of the AMND changes depending on the difference of the target base, the fluorescent strength is measured so that single nucleotide substitution can be detected. Since the AMND particularly selectively interacts with C (cytosine) as the target base, all single nucleotide substitutions (C/T, C/G, C/A) to which the C (cytosine) is related can be detected.

Here, when the DNA is mixed with the AMND, the AMND may be mixed in the form of solution including the AMND, or may be mixed in the form of powder or a solid. Further, the fluorescence may be visually measured by using a UV lamp or may be measured by using a device such as a fluorescence spectrophotometer, a fluorescence microscope, a densitometer, etc.

In this example, in order to inspect an effect of the detection of the single nucleotide substitution (C/G) by the AMND, a target DNA (a sequence a) of 23 mer and two kinds of detecting DNAs (sequences b and c) respectively of 11 mer

as described below were prepared as model sequences. Here, in the sequence a, S designates G (guanine) or C (cytosine).

(sequence a) 5'-TCTCCGCACACSTCTCCCCACAC-3' (sequence no. 1)

(sequence b) 5'-GTGTGCGGAGA-3' (sequence no. 2)

(sequence c) 5'-GTGTGGGGAGA-3' (sequence no. 3)

Specifically, in this example, 600 μM target DNA solution (the sequence a) of 25 μl as an object to be inspected, two kinds of 600 μM detecting DNA solutions (the sequences b and c) of 25 μl, 500 mM NaCl solution of 50 μl as an ionic strength conditioner, 50 mM sodium cacodylate solution of 50 μl including 5 mM EDTA as a pH buffer and 150 μM AMND solution of 50 μl were mixed together and MilliQ solution was added to the mixed solution to obtain a total quantity of 250 μl. An annealing process was carried out to the obtained DNA solution by a thermal cycler to measure the fluorescent strength. The fluorescence was measured by using a fluorescence measuring cell having an optical path length of 2 mm × 10 mm.

Fig. 4 shows a fluorescence spectrum when the target DNA is not added (DNA free) and fluorescence spectrums when the target base S of the target DNA is G (guanine) and C (cytosine). Here, excitation wavelength in Fig. 4 is 350 nm. As shown in Fig. 4, when the target base S is C (cytosine), the fluorescence is extremely quenched. This phenomenon may be considered to arise, because the AMND is stacked on the nucleobase adjacent the gap part and a stable combined

body is formed due to the formation of a hydrogen bond with the target base (C). In such a way, whether or not a quenching exists is detected, so that a user can know that the target base Y is G (guanine) or C (cytosine).

Fig. 5 shows a result obtained when the same DNA solution is put in a transparent tube made of polypropylene and the fluorescence is visually detected by using the UV lamp having the excitation wavelength of 350 nm. In Fig. 5, the fluorescence is also shown when the solution merely including the target DNA and the detecting DNAs and the solution merely including the AMND are respectively put into the transparent tubes. As shown in Fig. 5, when the target base S is C (cytosine), the fluorescence is extremely quenched and can be even visually recognized.

(Second Example)

In a second example, the same AMND as that of the first example was used as a receptor molecule to evaluate the adaptability to the detection of all single nucleotide substitutions (C/T, C/G, C/A) to which C (cytosine) is related.

In this example, in order to inspect an effect of the detection of the single nucleotide substitutions (C/T, C/G, C/A) by the AMND, a target DNA (a sequence d) of 107 mer and detecting DNAs (sequences e and f) respectively of 15 mer as described below were prepared as model sequences. Here, in the sequence d, N designates G (guanine), C (cytosine), A (adenine) or T (thymine).

(sequence d)

5'-CTATTGTTGGATCATATTCGTCCACAAAATGATTCTGAATTAGCTGTA TCGTCAAGGCACTCTTGCCTACGCCANCAGCTCCAACTACCACAAGTTT ATATTCAGTC-3' (sequence no. 4)

(sequence e) 5'-TGGCGTAGGCAAGAG-3' (sequence no. 5)

(sequence f) 5'-TGGTAGTTGGAGCTG-3' (sequence no. 6)

Specifically, in this example, to 5 μM target single-stranded DNA solution (the sequence d) of 5 μl as an object to be inspected, 5 μM detecting DNA solutions (the sequences e and f) of 5 μl were added, and further, 500 mM NaCl solution of 10 μl as an ionic strength conditioner, 50 mM sodium cacodylate solution of 10 μl including 5 mM EDTA as a pH buffer and 5 μM AMND solution of 5 μl were added and MilliQ solution was added to the mixed solution to obtain a total quantity of 50 μl. An annealing process was carried out to the obtained DNA solution by using a thermal cycler to measure the fluorescent strength. The fluorescence was measured by using a fluorescence measuring cell having an optical path length of 3 mm × 3 mm.

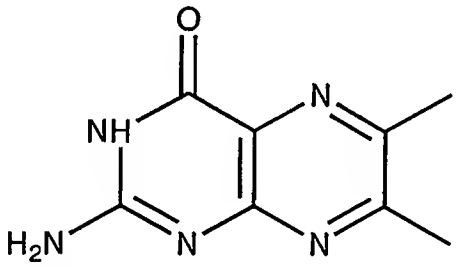
Fig. 6 shows a fluorescence quenching efficiency (%) when the target base N of the target DNA is G (guanine), C (cytosine), A (adenine) or T (thymine). Here, assuming that the fluorescent strength when the target DNA is not present is set to F_{free} and the fluorescent strength when the target DNA is present is set to F_{obs}, the fluorescence quenching efficiency is expressed by $((F_{free}-F_{obs})/F_{free}) \times 100$. Further, excitation wavelength in Fig. 6 is 350 nm and detected wavelength is 400

nm. As shown in Fig. 6, only when the target base N is C (cytosine), the fluorescence is extremely quenched. In such a way, whether or not a quenching exists is detected so that a user can know whether or not the target base N is C (cytosine). That is, the AMND is used as the receptor molecule so that all the single nucleotide substitutions to which C (cytosine) is related can be detected.

(Third Example)

In a third example, as a receptor molecule, 2-amino-6,7-dimethyl-4-hydroxypteridine (DiMe-pteridine) as a pteridine derivative as shown in a below-described chemical formula was employed.

Chemical formula 2



This DiMe-pteridine shows fluorescence emitting characteristics and interacts with a target base when the DiMe-pteridine is inserted into a gap part between two kinds of detecting DNAs as described below. Since the fluorescent strength of the DiMe-pteridine changes depending on the difference of the target base, the fluorescent strength is measured so that single nucleotide substitution can be detected. Since the DiMe-pteridine particularly selectively interacts with G (guanine) as the target base, all single nucleotide substitutions (G/C, G/A, G/T) to

which the G (guanine) is related can be detected.

In this example, in order to inspect an effect of the detection of the single nucleotide substitution (G/C, G/A, G/T) by the DiMe-pteridine, a target DNA (a sequence g) of 23 mer as described below and the above-described detecting DNA (the sequence b) of 11 mer were prepared as model sequences. Here, in the sequence g, N designates G (guanine), C (cytosine), A (adenine) or T (thymine). In the sequence g in this example, since sequences before and after the target base N are the same, one kind of the detecting DNA (the sequence b) of two equivalents was added to the target DNA (the sequence g) to form a gap part at a part opposed to the target base N.

(sequence g) 5'-TCTCCGCACACNTCTCCGCACAC-3' (sequence no. 7)

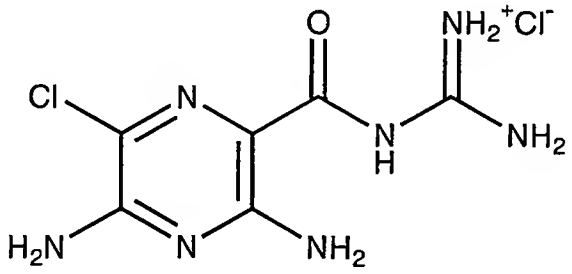
Specifically, in this example, to 5 μM target single-stranded DNA solution (the sequence g) of 10 μl as an object to be inspected, 10 μM detecting DNA solution (the sequence b) of 10 μl was added, and further, 500 mM NaCl solution of 10 μl as an ionic strength conditioner, 50 mM sodium cacodylate solution of 10 μl including 5 mM EDTA as a pH buffer and 1 μM DiMe-pteridine solution of 5 μl were added and MilliQ solution was added to the mixed solution to obtain a total quantity of 50 μl. An annealing process was carried out to the obtained DNA solution by using a thermal cycler to measure the fluorescent strength. The fluorescence was measured by using a fluorescence measuring cell having an optical path length of 3 mm × 3 mm.

Fig. 7 shows a fluorescence quenching efficiency (%) when the target base N of the target DNA is G (guanine), C (cytosine), A (adenine) or T (thymine). Here, excitation wavelength in Fig. 7 is 343 nm and detected wavelength is 435 nm. As shown in Fig. 7, only when the target base N is G (guanine), the fluorescence is extremely quenched. In such a way, whether or not a quenching exists is detected so that a user can know whether or not the target base N is G (guanine). That is, the DiMe-pteridine is used as the receptor molecule so that all the single nucleotide substitutions to which G (guanine) is related can be detected.

(Fourth Example)

In a fourth example, as a receptor molecule, amyloride (N-amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride) as shown in a below-described chemical formula was employed.

Chemical formula 3



This amyloride shows fluorescence emitting characteristics and interacts with a target base when the amyloride is inserted into a gap part between two kinds of detecting DNAs as described below. Since the fluorescent strength of the amyloride changes depending on the difference of the target base, the fluorescent

strength is measured so that single nucleotide substitution can be detected. Since the amyloride particularly selectively interacts with T (thymine) as the target base, all single nucleotide substitutions (T/G, T/C, T/A) to which the T (thymine) is related can be detected.

In this example, in order to inspect an effect of the detection of the single nucleotide substitution (T/G, T/C, T/A) by the amyloride, the above-described target DNA (the sequence g) of 23 mer and the above-described detecting DNA (the sequence b) of 11 mer were prepared as model sequences. Also in this example, the one kind of the detecting DNA (the sequence b) of two equivalents was added to the target DNA (the sequence g) to form a gap part at a part opposed to the target base N.

Specifically, in this example, to 5 μM target single-stranded DNA solution (the sequence g) of 10 μl as an object to be inspected, 10 μM detecting DNA solution (the sequence b) of 10 μl was added, and further, 500 mM NaCl solution of 10 μl as an ionic strength conditioner, 50 mM sodium cacodylate solution of 10 μl including 5 mM EDTA as a pH buffer and 1 μM amyloride solution of 5 μl were added and MilliQ solution was added to the mixed solution to obtain a total quantity of 50 μl. An annealing process was carried out to the obtained DNA solution by using a thermal cycler to measure the fluorescent strength. The fluorescence was measured by using a fluorescence measuring cell having an optical path length of 3 mm × 3 mm.

Fig. 8 shows a fluorescence quenching efficiency (%) when the target base N of the target DNA is G (guanine), C (cytosine), A (adenine) or T (thymine). Here, excitation wavelength in Fig. 8 is 361 nm and detected wavelength is 415 nm. As shown in Fig. 8, only when the target base N is T (thymine), the fluorescence is extremely quenched. In such a way, whether or not a quenching exists is detected so that a user can know whether or not the target base N is T (thymine). That is, the amyloride is used as the receptor molecule so that all the single nucleotide substitutions to which T (thymine) is related can be detected.

(Fifth Example)

In a fifth example, the same AMND as that of the first example was used as a receptor molecule to evaluate the adaptability to a PCR product.

In this example, in order to inspect the adaptability to the PCR product by the AMND, the above-described target DNA (the sequence d) of 107 mer and the above-described detecting DNAs (the sequences e and f) respectively of 15 mer were prepared as model sequences.

Here, the target DNA (the sequence d) in this example amplifies its antisense strand by a below-described forward primer (a sequence h) and a reverse primer (a sequence i) in an area including a codon 12 of a K-ras gene.

(sequence h) 5'-GACTGAATATAAACTTGTGG-3' (sequence no. 8)

(sequence i) 5'-CTATTGTTGGATCATATTCG-3' (sequence no. 9)

PCR solution was prepared with reference to a protocol of TaKaRa Taq

(produced by Takara Bio Inc.). PCR reaction solution has the following composition.

Forward primer 20 pmol (final concentration of 0.2 μM)

Reverse primer 300 pmol (final concentration of 3.0 μM)

Target DNA (the sequence d) 0.5 ng

TaKaRa Taq (DNA polymelase) 2.5 U

10 × PCR buffer 10 μl

2.5 mM dNTP 8 μl

These materials were mixed together in a 0.2 ml PCR tube, and further, MilliQ solution processed by an autoclave was added to the mixed solution to obtain a total quantity of 100 μl. Then, a PCR reaction was carried out in accordance with a protocol that the mixed solution was cooled to 4°C via processes carried out at 94°C for 5 minutes (94°C for 30 seconds to 52°C for 30 seconds to 72°C for 30 seconds) × 40 cycles to 72°C for 7 minutes. Thus, the target DNA (the sequence d) was amplified.

After the above-described PCR reaction was carried out, to the PCR reaction solution of 40 μl, a pH buffer (2 M sodium cacodylate, 33 mM EDTA, pH = 7.0) of 2.5 μl, 100 μM detecting DNA solution (the sequences e and f) respectively of 2.5 μl and 1 μM AMND solution of 5 μl were added to obtain a total quantity of 50 μl. The fluorescent strength of the obtained DNA solution was measured by using a fluorescence measuring cell having an optical path length

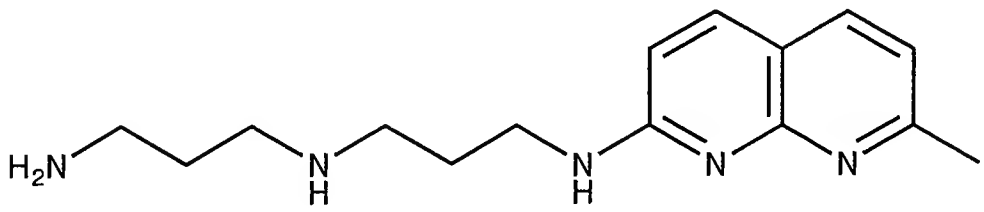
of 3 mm × 3 mm. A measuring temperature is 5°C.

Fig. 9 shows a fluorescence quenching efficiency (%) when the target base N of the target DNA is G (guanine), C (cytosine), A (adenine) or T (thymine). Here, excitation wavelength in Fig. 9 is 350 nm and detected wavelength is 400 nm. As shown in Fig. 9, only when the target base N is C (cytosine), the fluorescence is extremely quenched. In such a way, whether or not a quenching exists is detected so that a user can know whether or not the target base N is C (cytosine). Further, in this example, since an operation for removing DNA polymelase or dNTP or an accurate temperature control is not required, the PCR product can be rapidly and simply analyzed.

(Sixth Example)

In a sixth example, the detection of single nucleotide substitution by a surface plasmon resonance (SPR) method was evaluated. As a receptor molecule, AMND-DPA (N- (3-Amino-propyl) -N'- (7-methyl- [1,8] naphthyridin-2-yl) -propane-1,3-diamine) as shown in a below-described chemical formula was used to form a sensor chip (a micro-array) having the AMND-DPA fixed to a metal substrate.

Chemical formula 4



In the AMND-DPA, an alkyl chain having an amino group at its terminal end is introduced to a basic skeleton of the AMND to fix the AMND-DPA on the metal substrate and the AMND-DPA is synthesized from 2,6-diaminopyridine. When the AMND-DPA is inserted into a gap part between two kinds of detecting DNAs on the metal substrate, the AMND-DPA particularly selectively interacts with C (cytosine). At this time, since the signal strength of the SPR changes depending on the difference of the target base, the signal strength is measured so that all single nucleotide substitutions (C/T, C/G, C/A) to which the C (cytosine) is related can be detected.

In this example, in order to inspect an effect of the detection of the single nucleotide substitution by the sensor chip to which the AMND-DPA is fixed, the above-described target DNA (the sequence g) of 23 mer and the above-described detecting DNA (the sequence b) of 11 mer were prepared as model sequences. Also in this example, the one kind of the detecting DNA (the sequence b) of two equivalents was added to the target DNA (the sequence g) to form a gap part at a part opposed to the target base N.

Specifically, in this example, to 25 μM target DNA solution (the sequence g) of 10 μl as an object to be inspected, 20 μM detecting DNA solution (the sequence b) of 20 μl was added, and further, PBS-EP buffer (0.67 M phosphoric acid buffer solution, 1.5 M NaCl, 3 mM EDTA, 0.005 % Surfactant P20, pH = 6.4) was added thereto to obtain a total quantity of 500 μl. Further, an annealing

process was carried out to the obtained DNA solution by using a thermal cycler.

Further, as the sensor chip, a sensor chip CM5 (produced by Biacore AB) was used. The AMND-DPA was fixed to the sensor chip by using an amine coupling kit (produced by Biacore AB). Specifically, NHS (N-hydroxysuccinimide)/EDC (N-ethyl-N'-(3-dimethylaminoprpyl) carbodiimide hydrochloride) aqueous solution of 50 μl was injected to the sensor chip CM5 to activate a carboxyl group on the surface of the sensor chip by the NHS. Subsequently, AMND-DPA solution (diluted with 10 mM acetic acid buffer solution, pH = 5.5) of 0.20 mg/ml (0.73 mM) was injected to fix the AMND-DPA on the substrate. After that, 1 M ethanol amine aqueous solution of 50 μl was injected to block a remaining active NHS group. Further, the substrate was cleaned by using 8 mM NaOH aqueous solution of 60 μl.

Fig. 10 shows the signal strength (RU: Response Unit) of the SPR when the target base N of the target DNA is G (guanine), C (cytosine), A (adenine) or T (thymine). Here, an amount of the DNA solution used for measuring the SPR was 90 μl and the DNA solution was injected at a flow velocity of 30 μl/minute. Fig. 10 shows the signal strength of the SPR after 180 seconds subsequent to the injection. A quantity of fixed AMND-DPA is about 0.20 ng/mm^2 and a measuring temperature is 5°C.

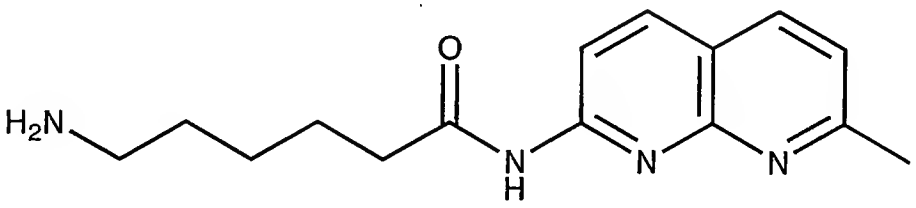
As shown in Fig. 10, when the target base N is C (cytosine), the signal strength of the SPR is maximum. This phenomenon may be considered to arise,

because the AMND-DPA is stacked on the nucleobase adjacent to the gap part and a stable combined body is formed due to the formation of a hydrogen bond with the target base (C), and consequently, a dielectric constant in the vicinity of the surface of the metal substrate changes. In such a way, the signal strength of the SPR is detected to know whether or not the target base N is C (cytosine). That is, the sensor chip to which the AMND-DPA is fixed is used so that all the single nucleotide substitutions to which the C (cytosine) is related can be detected.

(Seventh Example)

In a seventh example, the detection of single nucleotide substitution by a surface plasmon resonance (SPR) method was evaluated like the sixth example. As a receptor molecule, AcMND-C5A (6-Amino-hexanoic acid (7-methyl-[1,8]naphthyridin-2-yl)-amide) as shown in a below-described chemical formula was used to form a sensor chip having the AcMND-C5A fixed to a metal substrate.

Chemical formula 5



In the AcMND-C5A, an alkyl chain having an amino group at its terminal end is introduced to a basic skeleton of an AMND to fix the AcMND-C5A on the metal substrate and the AcMND-C5A is synthesized from 2,6-diaminopyridine.

When the AcMND-C5A is inserted into a gap part between two kinds of detecting DNAs on the metal substrate, the AcMND-C5A particularly selectively interacts with G (guanine). At this time, since the signal strength of the SPR changes depending on the difference of the target base, the signal strength is measured so that all single nucleotide substitutions (G/C, G/A, G/T) to which the G (guanine) is related can be detected.

In this example, in order to inspect an effect of the detection of the single nucleotide substitution by the sensor chip to which the AcMND-C5A is fixed, the above-described target DNA (the sequence g) of 23 mer and the above-described detecting DNA (the sequence b) of 11 mer were prepared as model sequences. Also in this example, the one kind of the detecting DNA (the sequence b) of two equivalents was added to the target DNA (the sequence g) to form a gap part at a part opposed to the target base N.

Specifically, in this example, to 200 μM target DNA solution (the sequence g) of 5 μl as an object to be inspected, 400 μM detecting DNA solution (the sequence b) of 5 μl was added, and further, PBS-EP buffer (0.67 M phosphoric acid buffer solution, 1.5 M NaCl, 3 mM EDTA, 0.005 % Surfactant P20, pH = 6.4) was added thereto to obtain a total quantity of 500 μl. Further, an annealing process was carried out to the obtained DNA solution by using a thermal cycler.

Further, as the sensor chip, a sensor chip CM5 (produced by Biacore AB)

was used. The AcMND-C5A was fixed to the sensor chip by using an amine coupling kit (produced by Biacore AB). Specifically, NHS/EDC aqueous solution of 50 μl was injected to the sensor chip CM5 to activate a carboxyl group on the surface of the sensor chip by the NHS. Subsequently, AcMND-C5A solution (diluted with 10 mM acetic acid buffer solution, pH = 5.5) of 1.0 mg/ml (3.7 mM) was injected to fix the AcMND-C5A on the substrate. After that, 1 M ethanol amine aqueous solution of 50 μl was injected to block a remaining active NHS group. Further, the substrate was cleaned by using 8 mM NaOH aqueous solution of 60 μl.

Fig. 11 shows the signal strength (RU: Response Unit) of the SPR when the target base N of the target DNA is G (guanine), C (cytosine), A (adenine) or T (thymine). Here, an amount of the DNA solution used for measuring the SPR was 60 μl and the DNA solution was injected at a flow velocity of 20 μl/minute. Fig. 11 shows the signal strength of the SPR after 180 seconds subsequent to the injection. A quantity of fixed AcMND-C5A is about 1.6 ng/mm^2 and a measuring temperature is 5°C.

As shown in Fig. 11, when the target base N is G (guanine), the signal strength of the SPR is maximum. This phenomenon may be considered to arise, because the AcMND-C5A is stacked on the nucleobase adjacent to the gap part and a stable combined body is formed due to the formation of a hydrogen bond with the target base (G), and consequently, a dielectric constant in the vicinity of

the surface of the metal substrate changes. In such a way, the signal strength of the SPR is detected to know whether or not the target base N is G (guanine). That is, the sensor chip to which the AcMND-C5A is fixed is used so that all the single nucleotide substitutions to which the G (guanine) is related can be detected.

As can be understood from the specific examples, according to the method for detecting a gene mutation in this embodiment, the double-stranded nucleic acid is formed by the single-stranded target nucleic acid 10 having the target base 11 composed of one or more continuous bases and the two kinds of single-stranded detecting nucleic acids 20a and 20b complementary to two kinds of partial sequences that sandwich the target base 11 between them. The receptor molecule 30 having the hydrogen bonding characteristics and the fluorescence emitting characteristics is added to the double-stranded nucleic acid to form the hydrogen bond with the target base 11. The fluorescent strength of the double-stranded nucleic acid bonded with the receptor molecule 30 is measured so that the gene mutation such as the single nucleotide substitution can be effectively detected.

Since a complicated operation such as labeling of the target DNA 10 as the object to be inspected or a heat control is not especially required, the number of processes is extremely small. Further, since the method for detecting a gene mutation does not depend, in principle, on the thermal stability of the double-stranded DNA itself, a very short time is merely necessary until the

detection and reproducibility is also excellent. Further, since a visual recognition using a UV lamp can be realized, the detection can be achieved under a state having no special equipment.

Further, the sensor chip (a micro-array) having many receptor molecules 30 or the detecting nucleic acids 20a accumulated on the substrate is manufactured and used as a kit for detecting a gene mutation. Thus, the detection with a high throughput that overcomes usual shortcomings can be realized.

In the above-described embodiment, the two kinds of detecting nucleic acids complementary to the two kinds of partial sequences that sandwich the target base between them are hybridized with the target nucleic acid to intentionally introduce the gap part. It is also known that the gap part is also produced during a restoring process of the DNA. (see a document "Erling Seeberg, Lars Eide and Magnar Bjoras, Trend. Biochem. Sci., 1995, 20(10), pp. 391-397".). Thus, as shown in Fig. 2, the double-stranded DNA solution can be dropped on the substrate to which the receptor molecule is fixed to react with the receptor molecule to detect whether or not the DNA is damaged.

Industrial Applicability

As described above, according to the present invention, the double-stranded nucleic acid is formed by the target nucleic acid and the two kinds of detecting nucleic acids to form a gap part at a position opposed to the target base. The hydrogen bond is formed by the receptor inserted into the gap part and the

target base so that a gene mutation such as the single nucleotide substitution generated in the target base can be effectively detected.

Further, the receptor or one of the two kinds of detecting nucleic acids is fixed to the substrate and the obtained body is used as a kit for detecting a gene mutation. Thus, the detection of a high throughput that overcomes the usual disadvantages can be realized.